Sari-Ak, D., Bahrami, S., Laska, M., Drncova, P., Fitzgerald, D. J., Berger-Schaffitzel, C., Garzoni, F., & Berger, I. (2019). High-Throughput Production of Influenza Virus-Like Particle (VLP) Array by Using VLP-factory™, a MultiBac Baculoviral Genome Customized for Enveloped VLP Expression. In R. Vincentelli (Ed.), *High-Throughput Protein Production and Purification: Methods and Protocols* (pp. 213-226). (Methods in Molecular Biology; Vol. 2025). Humana Press. https://doi.org/10.1007/978-1-4939-9624-7_10

Peer reviewed version

Link to published version (if available):
10.1007/978-1-4939-9624-7_10

Chapter 6: High Throughput production of influenza virus-like particle (VLP) array by using VLP-factory™, a MultiBac baculoviral genome customized for enveloped VLP expression

Running Head: HT Influenza VLP Array Production by MultiBac based VLP-factory™

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Abstract

Baculovirus-based expression of proteins in insect cell cultures has emerged as a powerful technology to produce complex protein biologics for many applications ranging from multiprotein complex structural biology to manufacturing of therapeutic proteins including virus-like particles (VLPs). VLPs are protein assemblies that mimic live viruses but typically do not contain any genetic material, and therefore are safe and attractive alternatives to life attenuated or inactivated viruses for vaccination purposes. MultiBac is an advanced baculovirus expression vector system (BEVS) which consists of an engineered viral genome that can be customized for tailored applications. Here we describe the creation of a MultiBac-based VLP-factory™, based

on the M1 capsid protein from influenza, and its application to produce in a parallelized fashion an array of influenza-derived VLPs containing functional mutations in influenza hemagglutinin (HA) thought to modulate the immune response elicited by the VLP.

Keywords

Baculovirus Expression Vector System (BEVS), small scale production, virus-like particle (VLP), MultiBac, Cre recombinase, Cre-LoxP fusion, influenza, hemagglutinin (HA).

Introduction

The inception of recombinant protein production technologies has had a decisive impact on both life sciences and drug discovery. Through recombinant overproduction, proteins that had been elusive before can now be produced in the quantity and quality required to decipher their structure and function. This set the stage for designing and validating intervention strategies to modulate their activity, which can now be translated into medications and therapies, through an elaborate process called the drug-discovery pipeline.

Recombinant protein overproduction also enables genetic engineering of complex protein specimens that themselves can be used as therapeutic drugs. For example, viruses contain protein materials, which typically enclose and protect the viral genetic information required by the virus for replication upon infecting an organism. Recombinant production of the protein shell components of a virus in the absence of its genetic material results in virus-like particles (VLPs). VLPs structurally resemble the live virus, but do not contain the genetic material which is essential for infectivity. Such recombinant VLPs are presently being used to vaccinate humans against numerous diseases including influenza [Fig 1], and also cancers caused by viral infection, a prominent example being cervical cancer caused by papilloma virus ***(1-6)***.

Research and development in academia and industry has long focused on small and isolated protein entities. Frequently, these are only the fragments or domains of a protein of interest, that can be efficiently produced by the recombinant host systems available, most typically the prokaryote *Escherichia coli*. Until today, expression in *E. coli* has dominated the protein production field. For example, >90% of all entries in the protein database (PDB) were produced in *E. coli*. For many therapeutic proteins, *E. coli*-based production has proven to be effective, efficient and relatively inexpensive. For example, the wide availability of *E. coli*–

produced recombinant human insulin, has revolutionized the treatment of patients suffering from diabetes, greatly reducing its cost and also alleviating allergic reactions hitherto occurring when insulin extracted from pigs or cows had been administered as a therapeutic ***(7)***.

Massive investment over the last 30 years into optimizing protein expression in *E. coli* has resulted in a large collection of genetically modified strains with widely diverse properties, each tailored to particular experimental needs. An enormous and growing assortment of circular DNA molecules (plasmids) exists for expression in *E. coli*, to enable the production of heterologous proteins encoded by genes which are inserted into these plasmids. *E. coli* expression has become commonplace in virtually every molecular biology laboratory in public and private research institutions ***(8-11)***.

The explosion of biological data which became available recently thanks to breakthroughs in genomics, proteomics and interactomics research, however, has revealed that the actual actors of cellular processes, particularly in eukaryotes, are not isolated proteins but rather complex multiprotein assemblies composed of up to a dozen or more protein subunits. Many of the individual eukaryotic proteins and most of the protein complexes, including therapeutically important assemblies such as VLPs cannot be produced efficiently in *E. coli*. The failure of the prokaryote *E. coli* to produce complex human protein specimens is not surprising given that eukaryotic cells and their component proteins are in general much more complex than prokaryotes and demand different folding, processing and post-translational modifications (i.e. decorations with small molecules such as sugars or phosphate groups conferring activity) that the *E. coli* protein production machinery cannot support. Consequently, eukaryotic protein expression systems have entered center-stage ***(12-16)***.

Among eukaryotic techniques, the baculovirus expression vector system (BEVS) has emerged as a particularly powerful method to produce complex protein biologics that are

authentically targeted and post-translationally processed. We introduced MultiBac, a modular baculovirus-based expression system particularly useful for producing protein specimens comprising many subunits ***(17-25)***. MultiBac consists of an engineered baculovirus from which we eliminated modalities detrimental to protein complex production. To facilitate the insertion of several to many protein encoding genes into the MultiBac virus, we created a set of small, circular DNA modules called Acceptor and Donor plasmids. Genes of interest can be inserted into these Acceptors and Donors by using ligation independent cloning procedures, and then combined into a multigene transfer plasmid by site specific recombination catalysed by Cre recombinase. The multigene transfer plasmid is then inserted by transposition into the MultiBac baculoviral genome which exists as a bacterial artificial chromosome in special *E. coli* cells. Moreover, we outfitted MultiBac by a further site specific recombination site directly inserted into the baculoviral genome. This site can be accessed by means of Cre-LoxP fusion reaction by further Donor plasmids which can contain genes encoding for even further subunits of a protein complex of choice, or, alternatively, modalities to modify the heterologous protein produced such as kinases, phosphatases, glycosylases and others ***(25-30)***. MultiBac is now in use in many laboratories (>1000) world-wide and contributed critically to accelerate ambitious research programmes in academia and industry ***(25)***.

In this report, we utilize a version of MultiBac which we designed for efficient expression of virus-like particles based on the influenza M1 capsid protein. We integrated a gene expressing M1 from H1N1 influenza strain into the viral backbone by site specific recombination, together with a fluorescent protein (mCherry) to monitor virus performance and VLP production [Fig 2]. Using this customized genome, VLP-factory^{TM}, we expressed in a parallelized fashion an array of influenza VLPs containing a series of mutations in a potentially immune-suppressive domain (ISD) within the influenza surface protein

hemagglutinin (HA). ISDs have been originally discovered in retrovirus surface proteins and recently also in influenza, and evidence suggests that the influenza ISD is identical or overlapping with the fusion peptide segment within HA ***(31)***. It has recently been shown that virus mediated membrane fusion is capable of inducing the innate immune responses in macrophages and dendritic cells ***(32)***. Apparently, the HA fusion peptide/ISD can thus inhibit this essential function of the immune system. For proper induction of an immune response in vaccines it is essential to maintain the membrane fusion capability of the HA, while removing its immune suppressive activity. Therefore, ISD mutations must be identified that on the one hand abolish immune suppressive activity, while maintaining membrane fusion activity. This is however no trivial task since the ISD is located on a structurally sensitive part of HA. The availability of an array of influenza VLPs containing randomized amino acids at defined positions in the ISD would be instrumental to identify such mutants. The influenza VLP variants, once produced from the array, comprising wild-type HA or HA mutants with randomized amino acids at defined positions in the HA ISD, can then be tested down-stream for membrane fusion activity (i.e. by hemolysis assay) and for eliciting and modulating immune response *in vivo* in animal models. Influenza VLPs containing mutations of the ISDs which may abolish the immune suppressive activity hold the promise to develop into VLP-based hyper-immunogenic antigens that could lead to broadly protecting influenza vaccines by eliciting a strong antibody titer upon immunization (in contrast to native HAs which are weak antigens).

We show here the production and characterization of influenza VLPs, facilitated by our customized MultiBac-derived baculoviral expression tool, VLP-factory^{TM}. VLP-factory^{TM} relies on the self-assembling, capsid-forming influenza M1 protein which we supply from the baculoviral genome in our expression experiments. Our VLP-factory^{TM}, however, is by no means restricted to the type of VLPs presented in this study. It is conceivable that most viral

envelope proteins that can be produced efficiently in insect cells infected with our customized baculovirus, will be efficiently incorporated in M1-based VLPs during the budding process. Thus, we anticipate that many other enveloped VLPs, from a wide range of viruses, can be produced efficiently by our approach, opening exciting avenues to produce potent VLP-based vaccines to combat viral disease.

1. Materials

Expression construct design is carried out *in silico* using a DNA cloning software of choice (i.e. VectorNTI, ApE, others). We used here gene synthesis for DNA encoding M1 from H1N1 influenza virus and hemagglutinin derived from both H1N1 and H5N1 influenza viral strains, and we used commercial mutagenesis services to introduce a set of potentially immune-modulating mutations in the hemagglutinin encoding genes. All internal restriction sites which may at some point be useful for subcloning into the MultiBac plasmids were eliminated by design, to facilitate possible future inclusion of further proteins such as NA and M2, or glycosylases to modulate the sugar structure of the heterologous proteins if desired ***(26-28)*** into our parallelized expression experiments. We applied codon optimization of the genes of interest for expression in insect cells by using the web-based algorithms provided at no cost from most synthetic DNA suppliers (e.g. www.idtdna.com/CodonOpt, OptimumGeneTM from www.genscript.com), concomitantly removing of potentially harmful RNA secondary structure elements in the transcripts. During codon optimization, we eliminated any restriction sites that are part of the so-called multiplication modules in the Acceptor and Donor plasmids to allow for flexibility of gene assembly if further proteins, for example other influenza factors or post-translational modifiers such as glycolysases would be co-expressed.

All reagents are prepared using ultrapure water (Millipore Milli-Q system or equivalent; conductivity of 18.2 MΩ·cm at 25°C) and analytical grade reagents. Buffers,

antibiotics and enzymes are stored at -20°C. We have described transfer plasmid generation and insertion into the MultiBac genome in detail elsewhere ***(18,19,33)***. Here we will therefore focus on the production, purification and analysis of the influenza VLP array we produced using our parallelized approach.

1.1 Materials for generating MultiBac-based VLP-factory™

1. Restriction endonucleases and reaction buffers (New England Biolabs, NEB).
2. T4 DNA ligase and buffer (NEB).
3. Cre recombinase enzyme and buffer (NEB).
4. Gel extraction kit (i.e. Qiagen, Germany).
5. Plasmid purification kit (i.e. Qiagen, Germany).
6. Regular *E. coli* competent cells (TOP10, HB101, or comparable).
7. *E. coli* competent cells containing *pir* gene (for Donor plasmids).
8. *E.coli c*ompetent cells DH10MultiBac harboring MultiBac baculoviral genome **(*18*)**.
9. An empty plasmid (pUCDM) for the insertion of genes encoding for influenza H1N1 M1 protein (GenBank ID ABD59883.1) and flurescent protein mCherry (GenBank ID ANO45948.1) via multiple cloning sites ***(18)***.
10. Antibiotics ampicillin, kanamycin, chloramphenicol, tetracyclin **(**for concentrations see Ref. ***18*)**.
11. Isopropyl β-D-1-thiogalactopyranoside IPTG (Sigma Aldrich).
12. Agar for pouring plates.
13. Media (LB, TB, SOC) for growing minicultures.

1.2 Materials for Generating influenza VLP producing baculoviruses

1. Sf21 or Sf9 insect cells.

2. Media (e.g. SF900 II SFM from Life Technologies or Hyclone form ThermoFisher) to grow insect cells.
3. Transfection reagent (e.g. FuGENE from Promega or JetPEI from Polyplus Transfection).
4. Sterile plastic ware (6-well tissue culture plates, Eppendorf pipettes).

1.3 Materials for producing influenza VLP array

1. Sf21 or Sf9 insect cell cultures.
2. Media (e.g. SF200SFM from Life Technologies or Hyclone form ThermoFisher) to grow insect cells.
3. Sterile glass ware (Erlenmayer shaker flasks, 250ml).
4. Sterile plastic ware (Eppendorf pipettes, sterile pipette tips).
5. Liquid nitrogen for freezing of cell pellets.

1.4. Materials for producing influenza VLP array

1. Phosphate-buffered saline (PBS) solution (Thermo Fischer Scientific).
2. Sterile Falcon or Greider tubes (15ml, 50ml).
3. Sterile ultracentrifugation tubes (Beckman).
4. Sucrose (Sigma Aldridge) solution (20%, 60%) in PBS, autoclaved or sterile filtered (20 μm nylon filter).
5. Sterile Eppendorf pipettes.
6. Unltracentrifugation equipment (SW28 rotor or comparable, ultracentrifuge).

2. Methods

The genes encoding for the influenza M1 and HA subunits and the functional mutants are designed *in silico,* and then inserted into the Donor and Acceptor plasmid of choice. Once designed, mono-, di- or, if more influenza factors such as NA and M2 are to be added, even

polycistronic expression cassettes can be created by a variety of means including DNA synthesis, restriction/ligation cloning, ligation independent cloning (LIC), sequence and ligation independent cloning (SLIC) or other methods such as Gibson cloning ***(34)*** as described in detail before ***(35,36)***, according to individual user preference. With the current highly competitive commercial offers we recommend custom DNA synthesis (*nota bene* including complete gene sequencing) to facilitate expression cassette construction, in particular for generation of mutant arrays as we carried out here for influenza HA.

2.1 Protocol for Generating VLP-factory™ baculoviral genome

1. Insert genes encoding for influenza H1N1 M1 protein (GenBank ID ABD59883.1) and fluorescent protein mCherry (GenBank ID ANO45948.1) into multiple cloning sites MCS1 and MCS2 of pUCDM plasmid ***(18)*** under polh and p10 promoter control, respectively, to yield pUCDM-M1-mCherry. Use generic restriction enzyme/ligation based cloning or ligation independent methods ***(33,34)*** as preferred.

2. Transform pir+ cells with pUCDM-M1-mCherry. Plate on agar containing chloramphenicol. Grow 25 ml bacterial culture as previously described ***(18)***. (**Note 1**)

3. Using standard plasmid preparation procedures, prepare 1 μg of pure pUCDM-M1-mCherry plasmid.

4. Prepare DH10MultiBac^{Cre} chemical competent cells containing the MultiBac baculoviral genome and expressing Cre recombinase enzyme according to published procedures ***(17)***.

5. Transform pUCDM-M1-mCherry plasmid into DH10MultiBac^{Cre} cells using standard salt-dependent transformation protocol, plate on agar plates containing tetracyclin,

kanamycin and chloramphenicol antibiotics As well as IPTG and BluOGal color reagent.

6. Pick a single blue colony and grow bacterial culture. Prepare competent cells and store in flash-frozen (liquid nitrogen) aliquots at -80°C. These are DH10VLP-factory™ cells containing the MultiBac-based VLP-factory [Fig 2].
7. Prepare VLP-factory Vo virus from an aliquot according to published procedures ***(19)***. Cells infected with VLP-factory™ virus turn brightly red due to mCherry expression.

2.2. Protocol for generating of influenza VLP array by using VLP-factory™

1. Transform DH10VLP-factory™ aliquots with transfer plasmid array encoding for wild-type and mutant influenza HA proteins and plate on agar containing kanamycin, gentamycin, tetracycline, IPTG and BluOGal.
2. Pick a single white colony from each transformation and prepare composite VLP-factory™ bacmid, followed by Vo initial virus generation using transfection reagent as described ***(19)***. Already at the Vo stage in 6 well plates, composite VLP-factory™ produced clones stain bright red [Fig 3].
3. Harvest budded VLP-factory™ virus producing influenza VLPs after 48-60h and amplify V1 virus in 250 ml shaker flasks containing 50 ml insect cell culture ***(19)***. Cell cultures turn red from 24-48h post infection. (**Note 2**)

2.3 Protocol for Influenza VLP purification.

1. About three days after proliferation arrest (3 dpa), the cell cultures will have a bright Bordeaux red color due to co-expressed mCherry [Fig 3]. Transfer cells in a sterile Falcon tube.
2. Spin at 4,000 rpm at RT in a table top centrifuge. (**Note 3**)

3. Repeat step 1 with fresh Falcon tube to avoid carrying over cell debris.
4. Ultracentrifuge cleared supernatant overnight at 26,000 rpm in an SW28 rotor (in centrifuge tubes containing 38 ml of supernatant each).
5. Gently resuspend the pellets in 100 μl PBS.
6. Prepare ultracentrifuge tube (38 ml volume) containing 20% sucrose and a cushion of around 2 ml of 60% sucrose at the bottom of the tube. (**Note 4**)
7. Load 1 ml of resuspended VLP pellet (from step 4).
8. Prepare identical tube with the same sucrose cushion as balance.
9. Ultracentrifuge overnight at 26,000 rpm in a SW28 rotor.
10. Remove 20% sucrose solution by gentle pipetting and collect the white layer between the two sucrose solutions (interface on top of the 60% cushion).
11. Load the white layer on a discontinuous 20-60% sucrose gradient.
12. Ultracentrifuge overnight at 26,000 rpm in SW28 rotor.
13. Collect white visible band containing purified VLP species and store at 4°C for further *in vitro* use including electron microscopy (EM) [Fig 4].
14. To remove sucrose for injection in animal models [Fig 5], repeat ultracentrifugation as above, carefully remove sucrose containing supernatant, and gently resuspend the pellet in sterile cold PBS (adjust concentration to the particle density required for injections).

4. Notes

1. The propagation of a Donor and its derivatives depends on cells that express the *pir* gene (such as PIR1 and PIR2 from Invitrogen). This is due to the conditional origin present on these plasmids ***(37)***. Acceptors and their derivatives (which we used here for the HA encoding genes), on the other hand, contain a common ColE1-derived

origin and can be propagated in any *E. coli* cloning strains (TOP10, TG1, DH10, HB101).

2. We processed batches of up to 25 different VLP array members using this approach, which allowed us to control logistics with ease. High-throughput approaches to culture insect cells as we described more recently could conceivably allow for parallel processing of many more specimens at the same time in smaller volumes in a table-top multi-fermenter ***(38)***.
3. All steps during influenza VLP purification are performed on ice. Sucrose solutions are prepared in PBS and sterile filtered through a 0.2 µm filter.
4. During influenza VLP production, if removal of sucrose is desired for down-stream processing (DSP), the white band can be diluted in PBS, ultra-centrifuged overnight at 26.000 rpm in an SW28 rotor. The pellet is then resuspended in PBS.

Acknowledgements

We thank all members of the Berger and Schaffitzel laboratories for helpful discussions, as well as Rob Ruigrok, Thibaut Crepin and Darren Hart for expert insight in influenza biology. We are grateful to Karin Huard for assistance with gradient preparation, and Yan Nie for introduction to negative-stain EM. This work was supported by the European Commission Framework Programme 7 projects ComplexINC (contract nr. 279039) and SynSignal (contract nr. 613879).

Competing financial interest statement

The authors declare competing financial interest. Parts of the technology here described are subject of international patent EP2403940 and licensed exclusively to Geneva Biotech SARL.

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Figure Legends:

Figure 1. Influenza Virus-like particles. Influenza virus structure, as deduced from electron microscopy (EM), is shown (left). The genetic material (green), vital for infectivity, is protected by an envelope made of proteins hemagglutinin (HA, colored in blue), neuraminidase (NA, colored in red) and matrix proteins (M1, colored brown; M2, colored in purple). HA and NA decorate the lipid membrane envelope. Synthetic influenza virus-like particles (VLPs) can be produced by recombinant protein overproduction (right). The VLPs can contain all envelope proteins as shown here, resulting in particles that are structurally virtually identical to the live virus with the important distinction being that all genetic material is absent from the VLPs, rendering the VLPs non-infectious and safe. Alternatively, VLPs can contain a subset of the envelope proteins, such as only hemagglutinin as presented in this study. Such VLPs are excellent candidates for vaccination. The influenza virus illustration was adapted from www.kimicontrol.com/microorg, courtesy of D. Jordan.

Figure 2: MultiBac-based VLP-factory^{TM}. The MultiBac baculoviral genome was customized for enveloped virus-like particle production by Cre-LoxP mediated insertion of a plasmid comprising expression cassettes for influenza H1N1 matrix protein M1 and fluorescent protein mCherry as a marker for tracking virus performance as shown schematically on the right. Insertion of transfer plasmids comprising expression cassettes for influenza surface proteins (hemagglutinin HA and/or neuraminidase NA) result in efficient expression of influenza virus-like particles (VLPs) as shown in EM images at the bottom. Recombinant influenza VLPs produced by the customized MultiBac genome (VLP-factory^{TM}) are virtually indistinguishable in size, shape and appearance from life influenza virus (inset, image courtesy of R. Ruigrok). Scale bars (50μm) are drawn in white.

Figure 3: Production of influenza VLP array comprising HA mutants. VLP-factory™ was utilized for producing an array of functional influenza VL variants. A transfer plasmid library encoding for wild-type and mutant influenza HA5 and HAB proteins was inserted in parallel into VLP-factory™ (top). Initial virus was prepared in 6-well plates already showing the characteristic red color resulting from expression of mCherry (below). Small-scale amplification in shaker-flasks (middle) resulted in sufficient cell pellet to extract and purify the influenza VLP array by sedimentation and gradient centrifugation for functional study (bottom).

Figure 4: Electron micrographs of purified influenza VLPs. A selection of VLP specimens is shown as analyzed by negative stain electron microscopy (EM). Production of M1 only by VLP-factory™ already results in globular shapes (top row) indicating efficient VLP formation in absence of influenza envelope protein. Co-expression of influenza HA (HA5, HAB) results in VLPs showing the characteristic pattern of protruding spikes (middle and bottom rows). Scale bars (50μm) are drawn in white.

Figure 5: VLP-factory™ produced influenza VLPs are functionally active. The presence of HA protein in a selected VLP preparation (HA5 M1) assayed by Western blot with specific antibody (H5N1 MIA-0052) is shown (left). The right lane in the Western blot contained VLPs comprising M1 only, and no HA signal is observed. M denotes molecular weight marker (kD). The recombinant influenza VLP was assayed in a hemolysis experiment (middle) showing up to 30% hemolysis of red blood cells (RBCs). VLPs containing M1 only did not cause hemolysis above natural level (red bar). The ELISA plot on the right shows antibody titers (IgG1) elicited after two injections (prime boost) of recombinant influenza VLP produced by using VLP-factory™, validating its protective potential against influenza infection in a murine model. PBS was used as a control.

Figures:

Figure 1:

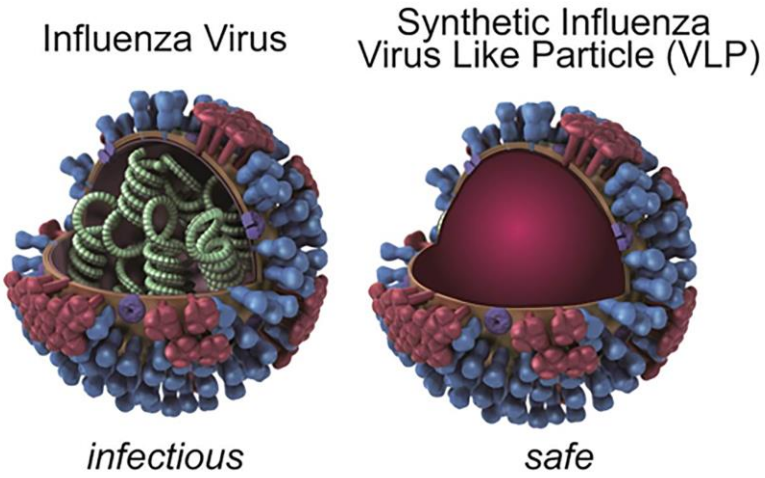


Figure 2:

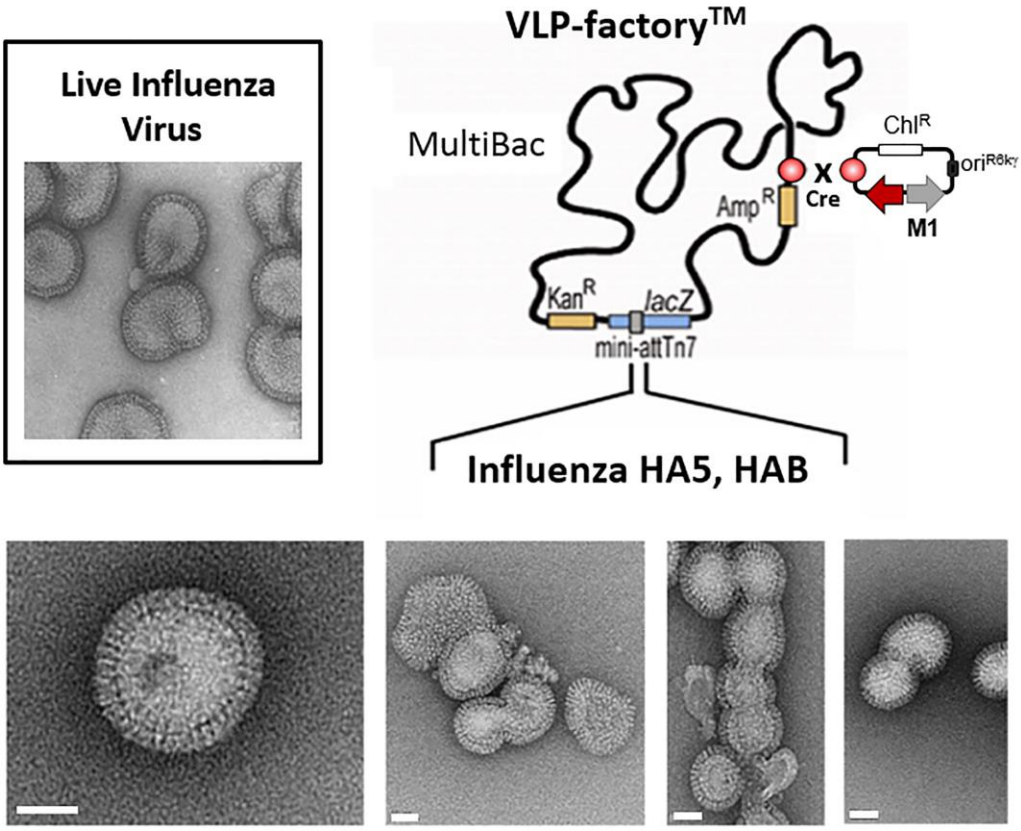


Figure 3:

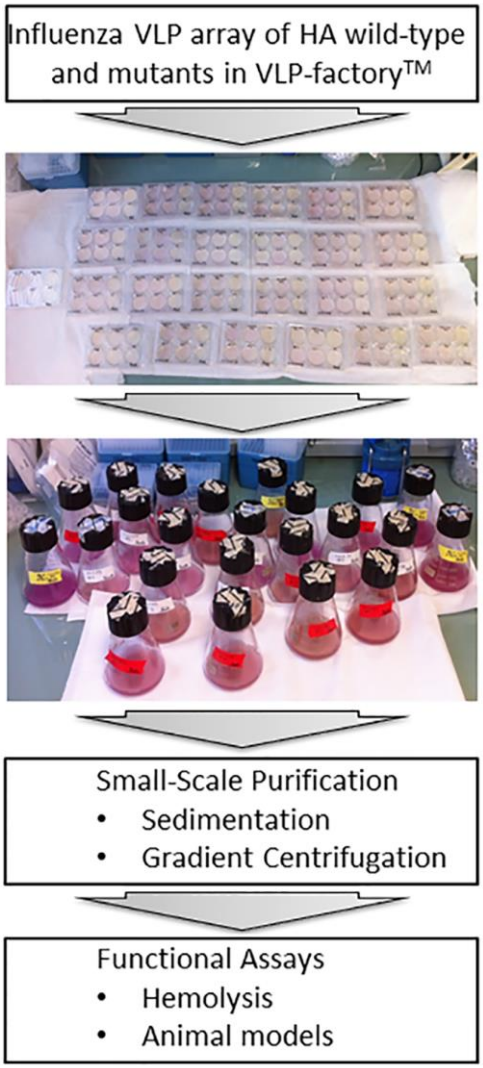


Figure 4:

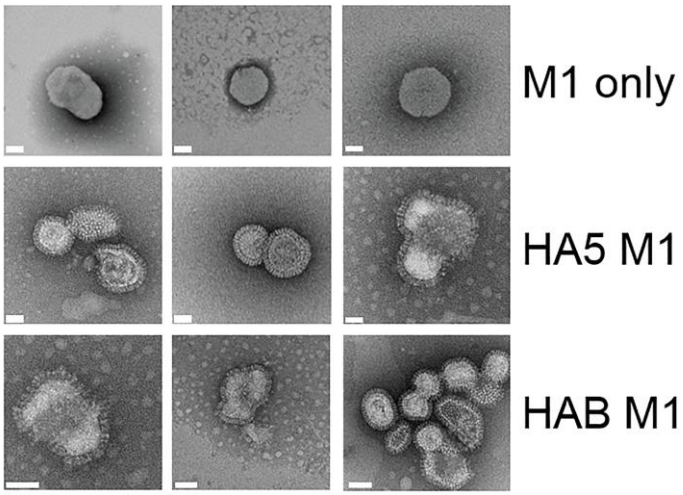
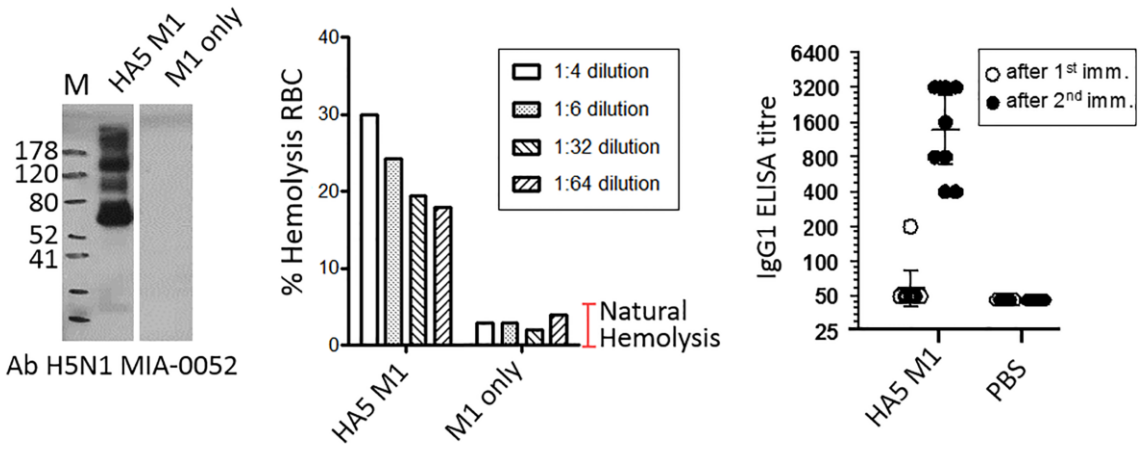


Figure 5:


M
HA5 M1
M1 only
178
120
80
52
41
Ab H5N1 MIA-0052
% Hemolysis RBC
40
30
20
10
0
1:4 dilution
1:6 dilution
1:32 dilution
1:64 dilution
Natural Hemolysis
HA5 M1
M1 only
IgG1 ELISA titre
6400
3200
1600
800
400
200
100
50
25
after 1st imm.
after 2nd imm.
HA5 M1
PBS